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Aneuploid strawberry (2*n* = 8*x* + 2 = 58) was developed from homozygous unreduced gamete (8*x*) produced by second division restitution in pollen

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ABSTRACT

Unreduced gamete formation is significant in the evolutionary development of complex polyploidy series found in wild strawberry, genus *Fragaria* (Rosaceae). Also, it is important for genetics and breeding in strawberry plants to elucidate the mechanism of unreduced gamete formation. The objective of this study was to search for ploidy anomalies resulting from artificial diploid × octoploid crosses, and examine the mechanism through which these unreduced gametes were produced. Five everbearing cultivars of *Fragaria vesca* L. diploid (2*n* = 2*x* = 14) were crossed with pollen from six June-bearing cultivars of *Fragaria* × *ananassa* Duch., octoploid (2*n* = 8*x* = 56). A total of 3000 mature seeds, 100 from each of the 30 parental combinations were sown at 23 °C/20 °C (day/night) under artificial lighting with a 16 h day. The seedlings were transplanted to pots and grown in a greenhouse. Reproductive and morphological observations, flow cytometry analyses, chromosome counts and DNA analyses using CAPS markers were performed to identify the genetic background of the offspring. Most of the seed (79%) did not germinate or died soon after germination. Of the seedlings produced, 7% seemed to be pure *F. vesca* based on morphological characteristics, flow cytometry analyses and chromosome counts; 14% were pentaploids (2*n* = 5*x* = 35), 0.1% were hexaploids (2*n* = 6*x* = 42), and 0.03% (one individual) was aneuploid (2*n* = 8*x* + 2 = 58). Electrophoresis banding patterns obtained by CAPS marker analysis were heterozygotic in the 8*x* pollen parent but homozygotic in the aneuploid progeny. Judging from the chromosome counts and the CAPS marker analysis, the aneuploid was the result of a homozygous unreduced pollen grain (8*x*) crossed with an incomplete chromosome compliment from the egg. Because of the homozygosity, the unreduced male gamete must have been derived from second division restitution (SDR) in the octoploid pollen parent.

1. Introduction

Cultivation of strawberry (*Fragaria* × *ananassa* Duchesne ex Rozier, 2*n* = 8*x* = 56) began in Europe more than 250 years ago with an accidental hybridization event between the octoploid wild species of *Fragaria chiloensis* and *Fragaria virginiana* (Darrow, 1966). It has become an extremely important fruit crop. With expansion of strawberry production into Europe and USA, various genetic studies were conducted using wild and cultivated strawberries from the early twentieth century. In those days, important findings of two kinds were performed to understand the evolution of the species in the genus *Fragaria*. The first finding was the existence of a euploid series of the wild species. Ichijima (1926) and Longley (1926) examined chromosomes of *Fragaria* and demonstrated the existence of diploid (2*n* = 2*x* = 14), hexaploid (2*n* = 6*x* = 42) and octoploid (2*n* = 8*x* = 56) wild species, and determined that the basic chromosome number was *x* = 7. About 20 wild species of 2*x*, 4*x*, 5*x*, 6*x*, 8*x*, and 10*x* have been identified (Staudt, 1989; Hancock, 1999; Hummer et al., 2009). Another finding was formation of the irregular polyploidy plants obtainable by hybridization between different species with different polyploidy levels. Yarnell (1931) reported that an enneaploid, i.e., nonaploid, plant (2*n* = 9*x* = 63) was obtained from *Fragaria vesca* crossed with *F. chiloensis*. Fedorova (1934) described two pentaploids obtained from the diploid *F. vesca* × *Fragaria moschata* L. (synonym = *Fragaria elatior* hexaploid (2*n* = 6*x* = 42)). Other researchers reported similar phenomena (Ichijima, 1926; Longley, 1926; Scott, 1951; Darrow, 1966; Morishita et al., 1996; Bors and Sullivan, 2005). Scott (1951) suggested that the emergence of polyploidy strawberries was associated with the production of unreduced gametes. These gametes

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are significant in producing high polyploidy levels of wild strawberry plants (Bringhurst and Senanayake, 1966; Darrow, 1966; Senanayake and Bringhurst, 1967; Bringhurst and Gill, 1970). Actually, Staudt (1984) observed single and double restitution in microsporogenesis of a F1 hybrid between *F. virginiana* and *F. chiloensis*. In addition, Shi et al. (2002) confirmed 2*n* and 4*n* unreduced gamete formation in *F. vesca* and *F. pentaphylla*, and that the 2*n* gamete was formed by abnormal division at metaphase II, in other words; second division restitution (SDR). Although it is important for evolution, genetics and breeding in strawberry plants to elucidate the mechanism of unreduced gamete formation, few papers related to examination of genetic background in artificially produced irregular polyploid strawberries.

Four types of unreduced gamete formation occur in plants, depending on the meiotic stage at which the nuclear restitution occurs: first division restitution (FDR), intermediate meiotic restitution (IMR), post-meiotic restitution (PMR) and second division restitution (Ramanna and Jacobsen, 2003). The first and fourth types apply to strawberries. In FDR, the spindles fail to form; homologous chromosomes do not separate, and are encased by the nuclear membrane. The chromosomes duplicate and separate in the second meiotic division. Offspring retain parental heterogeneity. For SDR, chromosome separation occurs during the first meiotic division and the subsequent duplication produces highly homozygous gametes. DNA analysis of the backgrounds of the parents and polyploid offspring can determine whether FDR or SDR is involved (Veilleux, 1985; Werner et al., 1992; Bretagnolle and Thompson, 1995; Bastiaanssen et al., 1998).

DNA analysis can determine genetic relations between parents and their progenies. Kunihisa et al. (2003, 2005, 2009) have developed CAPS markers to identify octoploid strawberry cultivars. These markers are single-genome specific and sufficient to visualize single allelic pair conditions of homozygotes and heterozygotes. CAPS markers include a pair of forward and reverse primers and a restriction enzyme with which the primers are able to clip single allelic pairs. In a homozygote A type (HMZ-A) DNA segments have specific base sequences that cannot be cut by the restriction enzyme, and are represented as a single electrophoretic band. In homozygote B-type (HMZ-B) DNA segments can be cut, and two smaller bands are produced. If the larger and the two smaller bands are identifiable, the DNA segments are regarded as heterozygote type (HTZ). CAPS marker analysis can thus detect genetic background of parents and their irregular polyploidy progeny of unreduced gamete origin, If the electrophoretic banding patterns obtained by the CAPS markers indicates heterozygocity and coincides with the 8*x* parent and the progeny, then the progeny must be derived from an unreduced gamete produced by FDR. If the banding pattern is heterozygous in the 8*x* parent but homozygote in the progeny, then the progeny must have been derived from the unreduced gamete by SDR.

For this reason, our objective was to examine unusual polyploid progeny of *F. vesca* × *F.* × *ananassa* to determine whether FDR or SDR was involved in the unreduced gamete production.

2. Materials and methods

2.1. Plant growth and measurement

Five diploid everbearing strawberry cultivars, *F. vesca* 'Alba', 'Alexandria', 'Baron Solemacher', 'Mignonette' and 'Reugen' were used as maternal and six June-bearing type octoploid Japanese cultivars, *F.* × *ananassa* 'Ai-berry', 'Asuka ruby', 'Hokowase', 'Nyoho', 'Sachinoka' and 'Toyonoka' were used as paternal parents. Cross-pollination was conducted using at least five flowers of each combination from late autumn 2007 to late winter 2008 in a greenhouse at the Faculty of Agriculture, Kagawa University. Approximately 100, fully-mature seeds from each combination were sown in a commercial sowing medium and germinated at 23 °C/20 °C day/night temperatures under a 16 h day length with artificial lighting of 100 μmol/m^2/s. Later, 627 healthy offspring were transplanted each to a 300 ml pot filled with commercial potting medium. They were then transferred to the greenhouse from late September and grown on under natural day-length conditions. For each plant, inflorescence and stolon production were observed for the three months following germination.

2.2. Flow cytometry analysis

A nuclear sample was obtained using the method reported by Mishiba et al. (2000) to identify polyploidy levels of the plants, Samples were prepared from approximately 1 cm^2 of fresh young leaves of the parental plants, the offspring and the rice (*Oryza sativa* L. 'Nihonbare') which was used as internal standard. Tissues were chopped 30 times using a razor blade in a plastic Petri dish with a 0.2 ml drop of commercial buffer solution (solution A of plant high-resolution DNA kit type P; Partec GmbH, Munster, Germany). Crude nuclear samples were incubated for 15 min at room temperature and were then filtered through 20 μm nylon mesh. A 1 ml staining solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP K-30 (Wako Pure Chemical Industries Ltd.), 0.1% (v/v) Triton X-100, 2 mg l^{-1} 4′6-diamidino-2-phenylin-dole (DAPI), was added to the filtered nuclear sample. It was then left for 2 min at room temperature. The samples were analysed using a flow cytometer (PAS; Partec GmbH).

2.3. Chromosome counts

The pretreatment and fixation procedures were conducted as described by Iwatsubo and Naruhashi (1989, 1991) and Nathewet et al. (2007, 2009). The root tip cells were collected from daughter plants in the early evening, pretreated with 2 mM 8-hydroxyquinoline solution for 1 h at room temperature, and subsequently transferred to a 4 °C refrigerator for 15 h. The root tips were then fixed in a 3:1 absolute alcohol:glacial acetic acid solution for 40 min at room temperature. The root tips were then softened using 1 N HCl solution at room temperature for 2 h and subsequently at 60 °C for 10 min, followed by brief rinsing in distilled water. A single treated root tip was placed on a glass slide with a drop of 1.5% lacto-propionic orcein solution and allowed to stand for a few minutes. A cover slip was placed on the root tip and was gently tapped using a pair of fine forceps. The slide was then warmed using a spirit lamp for a few seconds and the cover slip pressed very gently with a finger. Chromosomes were observed at 100× magnification under a light microscope. The well-spread chromosomes were photographed at the metaphase stage. Images were stored using a 3CCD camera (Olympus XD500; Olympus Corp. Japan) connected to a computer running FLVFS-FIS software (Olympus Corp.).

2.4. CAPS marker analysis

The parental 2*x* and 8*x* cultivars and their progeny with genomes of unreduced gamete origin were subjected to CAPS marker analysis according to Kunihisa et al. (2003, 2005, 2009). DNA was extracted from 100 mg of young leaf tissue and purified using a plant mini kit (DNeasy; Qiagen Inc.). The eight CAPS markers showing heterozygous electrophoresis band patterns in the 8*x* parental cultivar were selected: APX-*Mlu*I, APX4-*Taq*I, CHI-*Pvu*II, OLP-*Dde*I, PGPA-*Acc*I, PGPA-RsaI, PYDA-*Hae*III, PYDA-*Cfr*13I. Subsequently, PCR amplification was conducted in 0.02 ml containing 1–10 ng of extracted genomic DNA, 200 μM each of dNTP, 10 mM

Fig. 1. Examples of A and B type seedlings obtained from a cross between *Fragaria vesca* and *F.* × *ananassa*.

Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U Taq polymerase (Takara Bio Inc.) and 1 μM of each primer. Amplification was performed (TC-412; Techne Corp.) under the following program: 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C; and extra extension for 5 min at 72 °C. The amplified DNA solution was treated directly with 4 U of the appropriate endonuclease in a 0.01 ml volume. Polymorphism was detected by separating the whole volume of treated DNA on 1.5% agarose gel (1× TBE (Tris borate EDTA)) containing ethidium bromide and visualised under a UV transilluminator.

3. Results

Based on reproductive behavior (Fig. 1), the 627 seedlings were grouped into 204 plants of A type and 423 plants of B type (Table 1). The A-type plants produced inflorescences during the three-month period following sowing but the B-type plants did not. In contrast, the B-type plants produced stolons, whereas the A-type plants did not. The 204 A-type plants appeared to be typical *F. vesca* by the morphological characteristics of their flowers and by flow cytometry analyses (Fig. 2A). A chromosome number of 14 was confirmed for each of the A-type plants examined (Fig. 3A).

Flow cytometry separated the remaining plants into three types (B1, B2 and B3) by differences in the peak values of their relative DNA contents. Relative to rice, the 418 B1-type values were about 1.5-times higher (Fig. 2B), the 4 B2-type were about twice as high (Fig. 2C), and the one B3-type was about 2.7-times as high (Fig. 2F). The pedigree for the B3-type was 'Alba' and 'Hokowase'. The peaks of 'Alba' and 'Hokowase' were approximately 0.71-times and 2.4-times that of rice (Fig. 2D and E), respectively. The chromosome quantities of the B1-, B2-, and B3-type seedlings were, respectively, 35, 42, and 58 (Fig. 3B, C, and F). The chromosome quantities of 'Alba' and 'Hokowase' in the parental plants of B3 seedling were, respectively, 14 and 56 (Fig. 3D and E). With the exception of the PGPA-*Acc*I, every electrophoresis band pattern produced by all CAPS markers was HTZ in the pollen parent of 'Hokowase', and was HMZ-A or HMZ-B in the B3 seedling (Fig. 4 and Table 2).

4. Discussion

4.1. Flow cytometry analysis

Some researchers have used propidium iodide (PI) staining for flow cytometry (Nehra et al., 1991; Akiyama et al., 2001; Brandizzi et al., 2001), and mithramycin staining (Nyman and Wallin, 1992) to measure the cell nuclear DNA content. However, for strawberry plants, a few papers have reported the use of DAPI staining which is easier than either the PI or mithramycin staining.

The relative DNA contents of the cells using DAPI staining were sufficient to distinguish the polyploidy levels in the progenies. Very few strawberry studies have addressed analytical methods for flow cytometry systems with DAPI staining. The method used by Mishiba et al. (2000) for petunia plants was adjusted. The incubation time when the chopped leaves were mixed with the solution was tripled. Furthermore, the incubation time when the sample mixed DAPI solution was reduced to 1/10 to obtain an acceptable histogram (Fig. 2). Rice leaves were used as the internal standard because rice had a small relative DNA content, and showed good histograms

Table 1
Percentages of A and B type seedlings obtained by cross between *F. vesca* and *F.* × *ananassa*.

Seed and pollen parents	A type[a] (%)	B type (%)	Total number of A and B plants
'Alba' × 'Ai-berry'	88.6	11.4	35
'Alba' × 'Asuka Ruby'	43.8	56.3	32
'Alba' × 'Hokowase'	66.7	33.3	21
'Alba' × 'Nyoho'	7.0	93.0	43
'Alba' × 'Sachinoka'	2.8	97.2	36
'Alba' × 'Toyonoka'	33.3	66.7	27
'Alexandria' × 'Ai-berry'	100.0	0.0	42
'Alexandria' × 'Asuka Ruby'	5.9	94.1	17
'Alexandria' × 'Hokowase'	100.0	0.0	2
'Alexandria' × 'Nyoho'	9.3	90.7	43
'Alexandria' × 'Sachinoka'	0.0	100.0	38
'Alexandria' × 'Toyonoka'	13.3	86.7	15
'Baron Solemacher' × 'Ai-berry'	96.4	3.6	28
'Baron Solemacher' × 'Asuka Ruby'	0.0	100.0	21
'Baron Solemacher' × 'Hokowase'	0.0	100.0	5
'Baron Solemacher' × 'Nyoho'	0.0	100.0	40
'Baron Solemacher' × 'Sachinoka'	14.3	85.7	14
'Baron Solemacher' × 'Toyonoka'	0.0	100.0	3
'Mignonette' × 'Ai-berry'	0.0	100.0	1
'Mignonette' × 'Asuka Ruby'	100.0	0.0	8
'Mignonette' × 'Hokowase'	50.0	50.0	2
'Mignonette' × 'Nyoho'	8.3	91.7	12
'Mignonette' × 'Sachinoka'	9.1	90.9	11
'Mignonette' × 'Toyonoka'	100.0	0.0	4
'Reugen' × 'Ai-berry'	100.0	0.0	17
'Reugen' × 'Asuka Ruby'	17.6	82.4	34
'Reugen' × 'Hokowase'	66.7	33.3	3
'Reugen' × 'Nyoho'	3.2	96.8	31
'Reugen' × 'Sachinoka'	20.0	80.0	10
'Reugen' × 'Toyonoka'	28.1	71.9	32
Total			627

[a] A type seedlings produced inflorescences, but not stolons for the duration of three months after sowing. In contrast, B type seedlings produced stolons, but not inflorescences.

Table 2
Electrophoretic patterns obtained using CAPS markers in parental plants and 8*x* aneuploid progeny.

Maker	Electrophoretic patterns		
	2*x F. vesca* 'Alba'	8*x F.* × *ananassa* 'Hokowase'	8*x* aneuploid progeny
APX-*Mlu*I	nd[a]	HTZ[b]	HMZ-B
APX4-*Taq*I	HMZ-A	HTZ	HMZ-A
CHI-*Pvu*II	nd	HTZ	HMZ-A
OLP-*Dde*I	nd	HTZ	HMZ-B
PGPA-*Acc*I	HMZ-B	HTZ	HTZ
PGPA-RsaI	HMZ-A	HTZ	HMZ-A
PYDA-*Cfr*13I	HMZ-A	HTZ	HMZ-A
PYDB-*Hae*III	HMZ-A	HTZ	HMZ-A

[a] nd denotes not detected.
[b] HMZ-A, HMZ-B and HTZ respectively signify homozygote A type, homozygote B type and heterozygote.

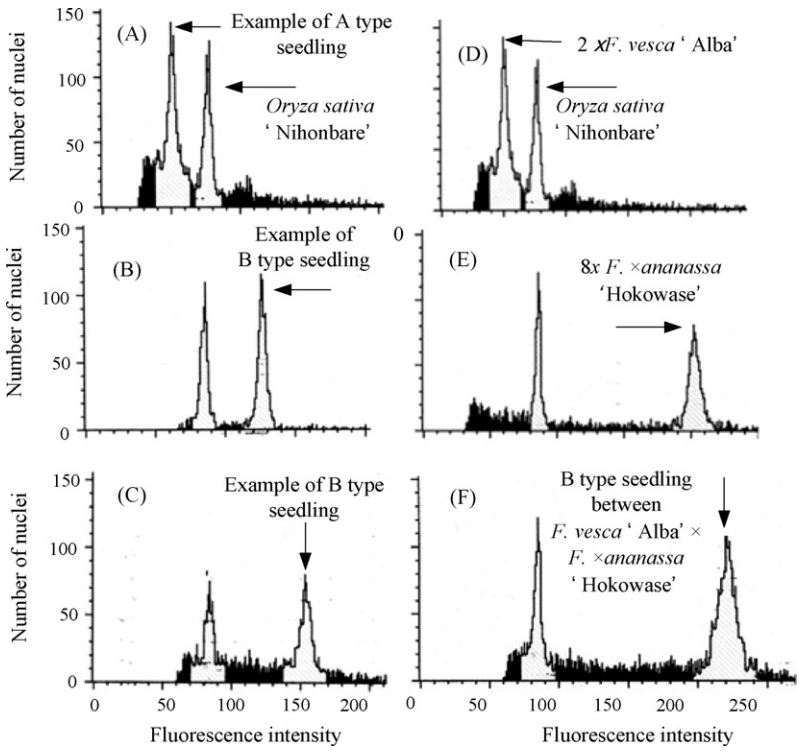

Fig. 2. Results of flow cytometry analysis. Panels denoted as A, B, C, and F show examples of results in A and B type seedlings. Panels D and E respectively portray were results of the parental diploid and octoploid of a progeny in F. Peaks shown as lines were obtained from rice used as the internal standard.

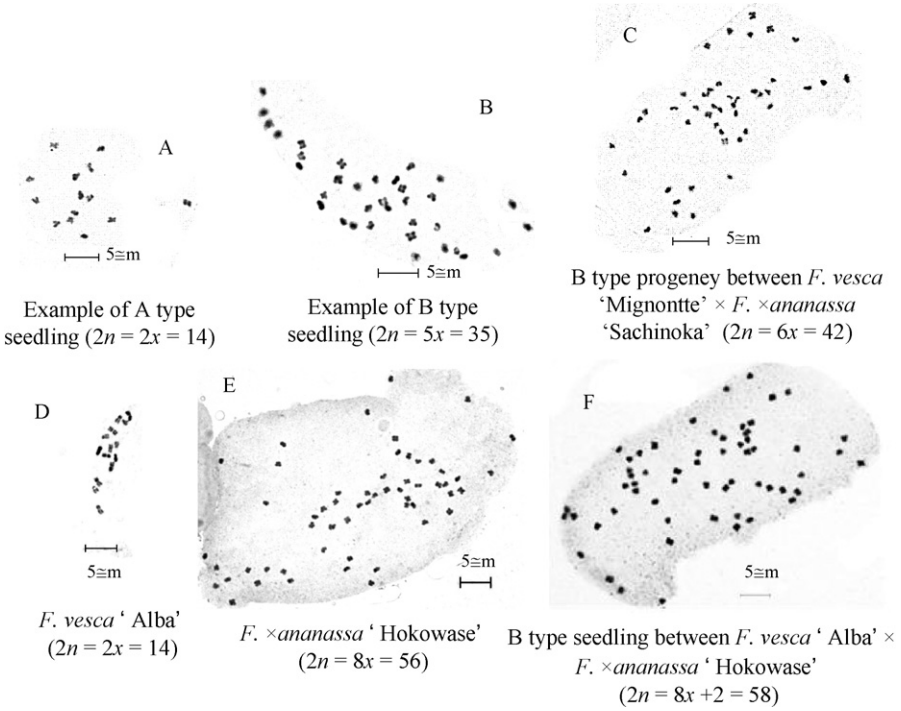

Fig. 3. Chromosome images of somatic cell. Panels denoted as A, B, C, and F show examples of A and B type seedlings. Panels D and E respectively depict chromosome images of the parental diploid and octoploid of a progeny (F).

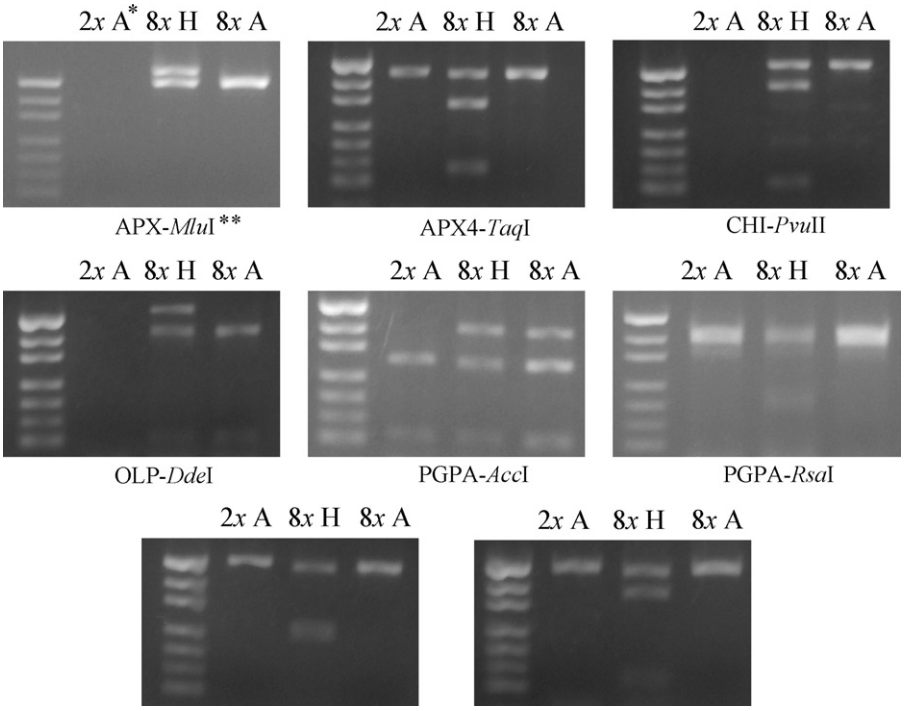

Fig. 4. Electrophoretic patterns produced using the CAPS method. *2*x* A, 8*x* H, and 8*x* A in the figure respectively denote the *F. vesca* 'Alba', *F.* × *ananassa* 'Hokowase', and the 8*x* aneuploid progeny between *F. vesca* 'Alba' and *F.* × *ananassa* 'Hokowase'. **Name of CAPS marker.

using the same procedure as that now developed for strawberry plants.

4.2. Combination of *F. vesca* × *F. virginiana*

Ichijima (1926) and Yarnell (1931) reported the production of octoploid progeny between *F. vesca* (2*x*) and *F. virginiana* (8*x*). In addition, Li et al. (2000) described emergence of octoploid interspecific hybrids between *F.* × *ananassa* and *F. vesca*, although the hybrids were identified as apomicts solely by their morphological characteristics. For these reasons, reciprocal cross-pollinations between *F.* × *ananassa* and *F. vesca* were conducted. However, for *F.* × *ananassa* (female) × *F. vesca* (male), only a few pentaploids were produced. These data were omitted from this study where everbearing (day-neutral) cultivars of *F. vesca* and June-bearing (short-day) cultivars of *F.* × *ananassa* were used as parents. Diploid plants have been documented from such crosses (Mangelsdorf and East, 1927; East, 1930, 1934; Ichijima, 1926; Yarnell, 1931; Morishita et al., 1996). These diploids were apomicts of the *F. vesca* plants, as indicated by Morishita et al. (1996), or possibly selfed plants.

4.3. Polyploidy levels of B1, B2, and B3 seedlings

Here, three types of seedlings were produced: B1, 418 pentaploids ($2n = 5x = 35$), B2, four hexaploids ($2n = 6x = 42$), and B3, one aneuploid ($2n = 8x + 2 = 58$), based the results of the flow cytometry analyses and chromosome counts. Considering the polyploidy levels in parental plants, the pentaploids (5*x*) were probably derived from combined reduced gametes ($x + 4x$) of parental plants. In addition, the hexaploids (6*x*) probably originated from the embryo composed of the unreduced *F. vesca* gamete (2*x*) and the reduced *F.* × *ananassa* gamete (4*x*). The aneuploid was apparently a hybrid that had originated from a combination of an incomplete reduced gamete (*x*) of *F. vesca* and the unreduced gamete (8*x*) of *F.* × *ananassa*. The five chromosomes might be diminished for the duration of initial growth. However, in the flow cytometry analysis, the peak value of relative DNA content in the aneuploid was similar to that of the enneaploid (9*x*), but not of the octoploid. For this reason, the plant root and leaf might be chimeral. Furthermore, Yarnell (1931) reported that the 8*x* progeny from a cross between *F. vesca* and *F. virginiana* seemed to be derived from a combination of the doubled unreduced gamete (4*x*) of *F. vesca* and the reduced gamete (4*x*) of *F. virginiana*. Considering that report, one possibility is that our aneuploid may have originated from the combination of the doubled unreduced gamete (4*x*) of *F. vesca* and the reduced gamete (4*x*) of *F.* × *ananassa*. Then two chromosomes split into four. However, this seems unlikely. The chromosomes in our aneuploid had a normal shape for strawberry chromosomes, as compared with previous results (Nathewet et al., 2009). Consequently, the aneuploid originated from an unreduced octoploid gamete.

4.4. Genetic background of the aneuploid of octoploid

CAPS marker analysis was conducted to clarify the genetic relations among parental cultivars of *F. vesca* 'Alba' (2*x*) and *F.* × *ananassa* 'Hokowase' (8*x*) and the aneuploid. The CAPS markers confirmed that the electrophoresis band pattern in 'Hokowase' was HTZ. The patterns of the aneuploid were HMZ-A or HMZ-B except for the PGPA-*Acc*I. In the case of the PGPA-*Acc*I, it was difficult to confirm whether the unreduced gamete (8*x*) form 'Hokowa' was HTZ or not. Because there was a possibility that the HTZ in the aneuploid of octoploid was composed of HMZ-A band produced by the gene introduced from the unreduced gamete (8*x*) of the 'Hokowaese' and HMZ-B bands did the gene introduced from the reduced gamete (x) in *F. vesca* as same as the parental plant of the *F. vesca* 'Alba'.

Therefore, from these results, the aneuploid must have been derived from the homozygous unreduced gamete of 'Hokowase' produced by SDR. It might be possible to produce an octoploid pure line at the level of nuclear DNA form the same combination of the parental plants. In addition, the production of unreduced gametes through SDR may have played a part in the evolution of polyploid strawberry species.

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